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**13. ABSTRACT (Maximum 200 Words)**

This project tests the hypothesis that Int6 regulates the 26s proteasome and acts as a tumor suppressor for breast cancer. We have accomplished a key step in this study by generating siRNA sequences that knock down Int6 expression, thus influencing mitosis. We have also prepared a good Int6 antibody and will soon learn whether Int6 associates with the proteasome biochemically. We have made great stride in exploring additional angels to better study Int6 functions. Firstly, we have evidence supporting an intriguing possibility that Int6 may be C-terminally truncated in human breast cancer cell lines. Secondly, our previous studies of the fission yeast system suggest that the tumorigenesis potential induced by Int6 inactivation can be counter-acted by oncogenic Ras. We have further investigated the relationship between Int6 and Ras and found that in fission yeast Int6 can colocalize with the Ras-Cdc42 pathway in the endomembrane. We will determine whether this occurs in human cells and ascertain its role in tumorigenesis.

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## INTRODUCTION

The *int6* gene was first isolated in a screen using the mouse mammary tumor virus (MMTV) as an insertional mutagen to seek genes that are important for breast cancer formation. Its molecular function, however, remains unknown. Our preliminary data derived from the study of an Int6 homolog in the fission yeast *Schizosaccharomyces pombe* support a hypothesis that Int6 regulates the function of the 26S proteasome, which functions to degrade growth regulatory proteins in an ubiquitin-dependent fashion. We envision that when Int6 functions are inactivated, the proteasome may be weakened to lead to accumulation of regulatory proteins, some of which are key players in the control of cell cycle and chromosome segregation. Deregulation of the cell cycle and chromosome stability are powerful factors in tumorigenesis. Our specific aims are to investigate whether altering Int6 function can influence (1) proteasome functioning and (2) chromosome segregation in human mammary epithelial cells.

## REPORT BODY

### Task 1: Generate cell lines with reduced Int6 levels

#### a. Int6 antibody production

Last year, we reported that we have generated a polyclonal antibody by using a peptide corresponding to the N-terminus of the Int6 as antigen and that this antibody can recognize Int6 when it is expressed in *S. pombe*. We are glad to report that we now have evidence that this antibody can efficiently detect Int6 protein from human cell lines, including that of the "normal" mammary epithelial cells, MCF10A (Figure 1). Thus we have accomplished the goal of this task to obtain our own anti-Int6 antibody, which is now named Int6-N1.

#### b. and c. Test candidate DNA sequences for Int6 knock-down

Last year, we made three siRNA expression vectors by using the pSUPER vector, but none successfully knocked down Int6 expression in HeLa cells. We thus decided to synthesize siRNAs for direct transfection. Furthermore, while we were getting ready for this experiment, Morris and Jalinot (Morris and Jalinot, 2005) published two siRNA sequences (I6.1 and I6.3), which can knock down Int6 expression by approximately 50% in HeLa cells. As shown in Figure 2, ours, as well as their siRNAs, all down-regulated Int6 expression in HeLa cells, and we were able to knock it down by more than 80%.

While we plan to screen more siRNA sequences to increase the pool of effective siRNA sequences using the HeLa cell system, we have started examining these tested siRNAs on human mammary epithelial MCF10A cells. Our preliminary data show that our siRNA (IN550), as well as one of the siRNA from the Jalinot group (I6.3), was able to knock down Int6 expression by approximately 50% (Figure 2), which is less than what we have accomplished in HeLa cells. We are currently investigating whether this is due to differences in transfection efficiencies or regulation of Int6 expression between cell lines. Furthermore, we are building expression vectors to determine whether they can produce effective siRNA to knock down Int6 expression, which is critical for establishing stable clones for detail and extensive phenotypic analyses.

### Task 2: Analyze proteasome function in engineered cells

While it is more desirable to perform functional analyses of cells, in which the level of Int6 is stably reduced, we are planning to analyze the transiently transfected HeLa cells as mentioned above to determine whether their sensitivities to proteasome inhibitors, such as MG132, have been altered.

As an alternative, as stated in the last progress report, we have established a stable MCF10A cell line, which expresses the cMYC-tagged Int6 (Figure 1). We are currently establishing an immunoprecipitation protocol to determine whether proteasome subunits can form complexes with Int6. The Rechsteiner lab has kindly provided us with antibodies that recognize many subunits in the proteasome (Gorbea et al., 2004). Furthermore, as shown in Figure 1, we have now a good polyclonal antibody against Int6, and are also using it to perform immunoprecipitation experiments to increase the chance of success.

**Task 3: Analyze genetic stability in engineered cells**

Based on our published fission yeast data, we propose that Int6 inactivation can weaken the proteasome, leading to genetic instability due to accumulation of mitotic regulatory proteins, such as securin (Yen et al., 2003). Consistent with this, as shown in Figure 3, in Int6-reduced HeLa cells, we frequently detect multinucleated cells, which appear to have undergone mitosis with a tripolar spindle. Morris and Jalinot (2005) made similar observations. We are currently examining Int6-reduced MCF10A cells for any signs of mitotic abnormalities.

Morris and Janilot (2005) did not examine proteasome functions in Int6-reduced HeLa cells. They did analyze the steady state levels of securin in these cells and found that they are not substantially different from those of untreated cells. Securin degradation has been thoroughly studied in *S. pombe,* in which it is degraded in a short time window in mitosis (Funabiki et al., 1996). If securin degradation is similarly dependent on the cell cycle in mammalian cells, it may be unproductive to simply measure total protein levels in an asynchronized culture. Potentially great variations in gene knock-down efficiencies may further complicate this type of study. To address this, we plan to first take a block-release strategy to synchronize Int6-reduced HeLa or MCF10A cells. Next, we will examine (1) steady-state securin protein levels, (2) steady-state levels of polyubiquitinated securin, and (3) half-lives of securin. We will repeat these experiments on a stable clone of Int6-reduced cell line to eliminate the issue of heterogenous efficiencies in gene knock-down.

**Additional progress**

In addition to experiments proposed in this project, we have made progress in other areas related to the Int6 study.

1. Functional analysis of the PCI domain in Int6.

At the C-terminus of Int6, there is a PCI domain, which is truncated in the mouse tumors by MMTV insertion, and which may play a role in interacting with the proteasome. After we found that our antibody can efficiently detect Int6 proteins in MCF10A cells, we started investigating whether there may be any changes in the structure of Int6 in human breast cancer cell lines. Intriguingly, our Int6N1 antibody, which recognizes a region in the N-terminus of Int6, detected a smaller protein only in tumor cell lines, but not in the control MCF10A cells (Figure 4). By contrast an antibody that recognizes the C-terminus of Int6, which is recently commercially available, did not detect these proteins. This observation taken at its face value suggests that Int6 may be frequently truncated in the C-terminus in human breast cancer cell lines. We are making another antibody that recognizes another region in the N-terminus to further examine this possibility. If successful, we will expand this study to examine human breast tumor tissues and analyze their genomic DNAs for the presence of tumor-associated frame shift mutations in *int6*.

2. Int6 proteins may localize to the endomembrane to interact with Ras G-proteins.

We have reported that the proteasome and mitotic defects associated with Int6 inactivation in yeast can be rescued by the presence of an oncogenic Ras protein (Yen et al., 2003). This and other data support a model in which Int6 and Ras can act cooperatively to regulate mitosis and proteasome functions.

The classic view of Ras signaling suggests that these proteins signal from the plasma membrane (PM) to mediate extracellular signals. However before reaching the PM, Ras must be covalently modified (lipidation) and localize to the endomembrane (e.g., ER and Golgi). Increasing evidence from the mammalian system argues that Ras signaling may be compartmentalized in the cell, which allows a given Ras protein to perform cell compartment-specific functions. For example, Ras proteins that are experimentally restricted to the endomembrane can be activated in the cell and stimulate known effectors (Chiu et al., 2002). We examined this idea in fission yeast, which is an ideal and simple system to investigate this. Fission yeast has a single Ras protein, Ras1, but it still controls two conserved pathways whose outputs are easily scored — a MAP kinase pathway to regulate mating, and a Cdc42 pathway to regulate cell polarity and interaction with Int6 and the proteasome. In a paper submitted for review, we have demonstrated that Ras1 regulates these two pathways at two cell compartments. That is, PM-restricted Ras1 selectively activates the MAP kinase pathway, while endomembrane-restricted Ras1 specifically activates the Cdc42 pathway. As such, our results have provided the most unambiguous evidence so far for compartmentalized Ras signaling.

In our published work, we found that Int6 specifically interacts with the Ras-Cdc42 pathway, but not the Ras-MAP kinase pathway; thus, we determined whether Int6 or its associated proteins in fission yeast localized to the endomembrane by sucrose gradient centrifugation. We have first examined the localization of an evolutionarily conserved Int6-binding protein, Moe1 (Chen et al., 1999), and found that it colocalizes with Ras that is targeted specifically to the endomembrane and with an ER marker, 13g6 (Figure 5), suggesting that Moe1, if not Int6 itself, localizes to the endomembrane where it interacts with the Ras-Cdc42 pathway. We are currently further testing whether fission yeast Int6 also associates with Ras and ER markers in sucrose gradient and preparing GFP-tagged expression vector to examine Int6 localization in mammalian cells.

**KEY RESEARCH ACCOMPLISHMENTS:**

1. We have obtained polyclonal anti-Int6 antibody and tested its efficacy in human cells (Figure 1).

2. We have created generated several siRNA sequences that can transiently knock-down Int6 expression in both HeLa and MCF 10A cells (Figure 2).

3. We have begun examining cell functions in Int6-reduced HeLa cells and found evidence of abnormal mitosis due to the presence of tri-polar spindle (Figure 3).

4. We have evidence suggesting that Int6 proteins may be C-terminally truncated in human breast cancer cell lines (Figure 4).

5. Our localization data suggest that Int6 may colocalize with Ras in the endomemebrane (Figure 5).

**REPORTABLE OUTCOMES:**

**1. One polyclonal anti-Int6 antibody.**

**2. Two siRNA sequences for down regulating Int6 expression in human mammary epithelial cells.**

**3. Papers**


1. **Chang, E. C.** and Schwechheimer, C. 2004. ZOMES III: the interface between signaling and proteolysis. *EMBO Rep.*, 5: 1041-1045.
2. Sap, J. and **Chang, E. C.** 2005. Int6. *AfCS-Nature Signaling Gateway _ Molecule Pages* (doi:10.1038/mp.a003493.01).
3. Onken, B., Weiner, H., Philips, M. and **Chang, E. C**. 2005. Compartmentalized signaling of Ras in

fission yeast. *Nat. Cell Biol.* Submitted.

**4. Meeting Abstracts:**

1. Yen, S. and **Chang, E.** 2004. Int6 — a link between tumorigenesis and proteasome functions. 2nd International Conference on Ubiquitin, Ubiquitin-like Proteins, and Cancer. M.D. Anderson Cancer Center.
2. Yen, S., Gordon, C., and **Chang, E.** 2004. Int6 _ a PCI protein that regulates tumorigenesis and the proteasome. ZOME III International Conference, Berlin, Germany.
3. Yen, S., Gordon, C. and **Chang, E.** 2004. Int6 _ a link between tumorigenesis and the proteasome. American Society for Biochemistry and Molecular Biology meeting in Boston.
4. Onken, B., Philips, M. and **Chang, E. C.** 2004. Fission yeast Ras localizes to different cell compartments to selectively regulate different signaling pathways. 3rd Internal Fission Yeast Meeting at San Diego.
5. Zhe, S., Cabrera, R., and **Chang, E. C.** 2004. Regulation of proteasome import and assembly by Int6/Yin6. 3rd Internal Fission Yeast Meeting at San Diego.
6. Onken, B., Philips, M. and **Chang, E. C.** 2004. Fission yeast Ras localizes to different cell compartments to selectively regulate different signaling pathways. Research Retreat at the Institute of Molecular Biology, Academica Sinica, Taiwan.

**CONCLUSION:**

This project tests the hypothesis that Int6 regulates the 26s proteasome and acts as a tumor suppressor for breast cancer. A key step in this study is to knock-down Int6 expression. We have made good progress in this and have identified several siRNA sequences, and in the future we will focus on creating stable cell lines in which Int6 expression is knocked down. Before this is ready, however, we plan to examine several key cell functions in cells whose Int6 is transiently down-regulated, and to perform biochemical experiments to determine whether Int6 can associate with the proteasome in human mammary epithelial cells. In addition to the proposed studies, we have made great stride in exploring and expanding our analysis of Int6 functions. Firstly, we have evidence supporting an intriguing possibility that Int6 may be C-terminally truncated in human breast cancer cell lines. In the future, we need to first verify this observation by examining Int6 using another antibody, which we are now preparing to recognize another region in its N-terminus, to make sure that those "shorter" proteins are indeed derived from full-length Int6 protein. If successful, we will further investigate whether such truncation can be found in human tumor tissues and uncover how this may occur. Secondly, studies of the *S. pombe* system suggest that the tumorigenesis potential induced by Int6 inactivation can be rescued by the presence of oncogenic Ras and this idea may explain why oncogenic mutations in *ras* genes are rare in breast cancer. We have now evidence that Int6 can colocalize with Ras in the endomembrane, and will determine whether this occurs in human cells and ascertain its role in tumorigenesis.

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Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., 2nd, Cox, A. D., and Philips, M. R. (2002). Ras signalling on the endoplasmic reticulum and the Golgi. Nat Cell Biol *4*, 343-350.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T., and Yanagida, M. (1996). Cut2 proteolysis required for sister-chromatid seperation in fission yeast. Nature *381*, 438-441.

Gorbea, C., Goellner, G. M., Teter, K., Holmes, R. K., and Rechsteiner, M. (2004). Characterization of mammalian Ecm29, a 26 S proteasome-associated protein that localizes to the nucleus and membrane vesicles. J Biol Chem *279*, 54849-54861.

Morris, C., and Jalinot, P. (2005). Silencing of human Int-6 impairs mitosis progression and inhibits cyclin B-Cdk1 activation. Oncogene *24*, 1203-1211.

Yen, H. C., Gordon, C., and Chang, E. C. (2003). Schizosaccharomyces pombe Int6 and Ras homologs regulate cell division and mitotic fidelity via the proteasome. Cell *112*, 207-217.

**APPENDIXES:**

Figures 1-5.

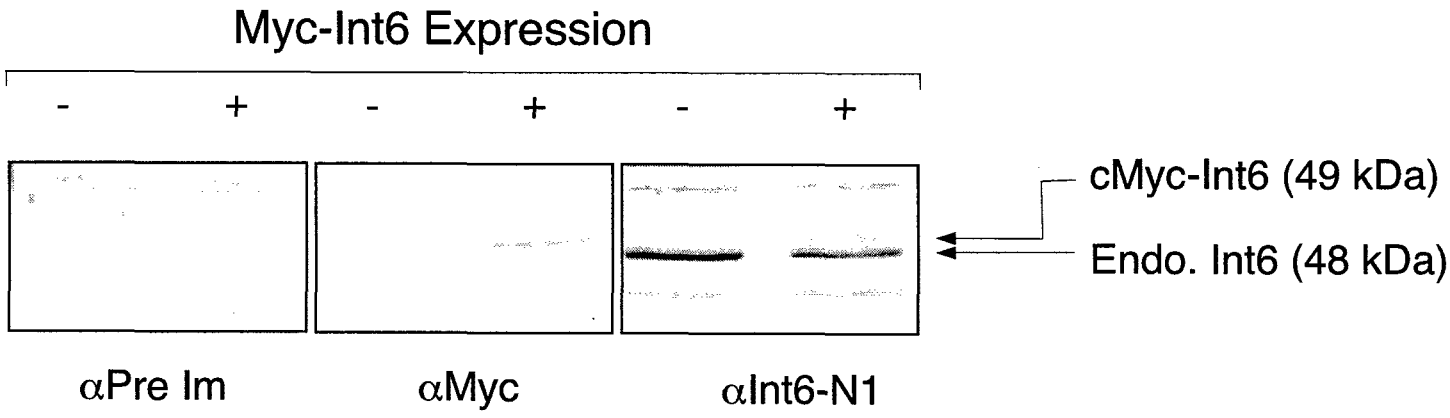


Figure 1: Polyclonal antibody that recognizes Int6 in human mammary epithelial cells. Cell lysates were prepared from MCF10A cells stably ectopically express c-MYC tagged Int6 and analyzed by Western blots using antibodies indicated at the bottom. Our antibody, named Int6-N1, detected both the endogenous Int6 protein as well as the cMYC tagged version. The same antibody also detects Int6 in HeLa and NIH3T3 cells (not shown).

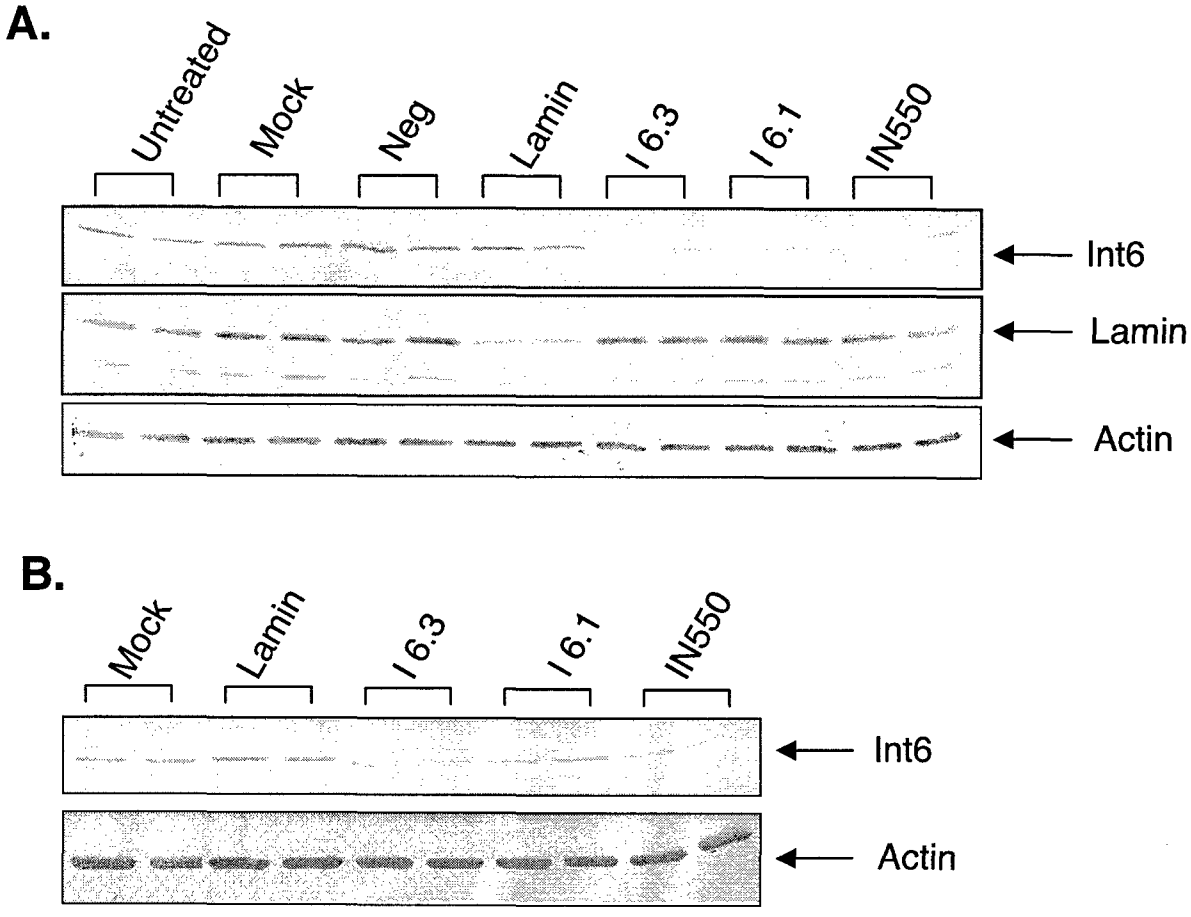


Figure 2. Knock-down of Int6 expression using siRNA. (A) HeLa cells in duplicates were either untreated or directly transfected by buffer only (Mock), nonspecific RNA (Neg,), siRNAs against lamin (Lamin) and siRNAs against Int6 (I6.3, I6.1, and IN550), and the cell lysates were analyzed by Western blots. β-actin was examined as a loading control. (B) MCF10A cells were tested similarly as in (A), but only selected samples are shown.


Figure 3. Abnormal mitotic cells when Int6 expression is knocked down. HeLa cells in which Int6 expression has been reduced by siRNA were stained to reveal DNA, and cells with three nuclei (arrow) can be readily detected.

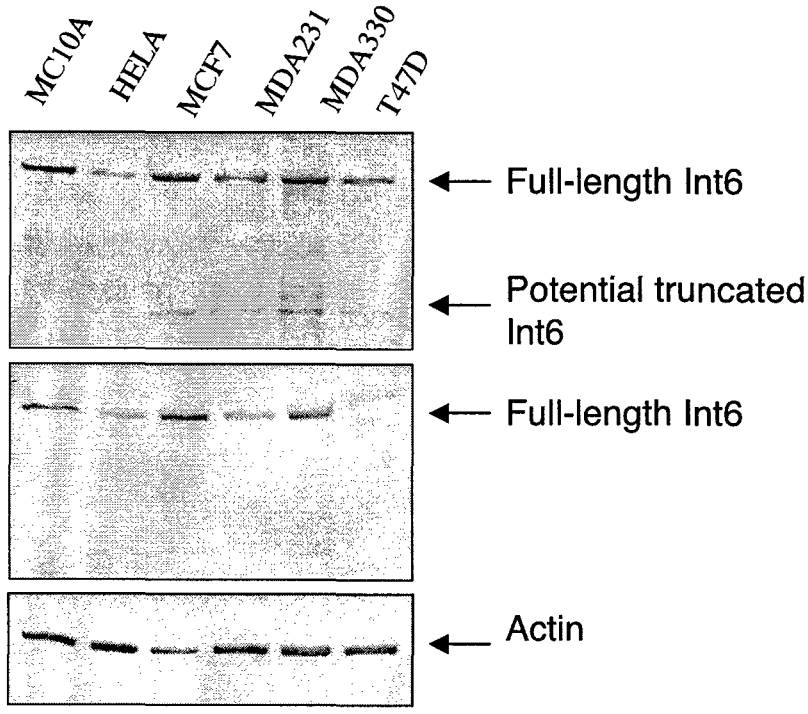


Figure 4. Detection of potential C-terminally truncated Int6 proteins in human transformed and breast cancer cell lines. Cell lysates were examined by either Int6N1, which recognizes the N-terminus of Int6 (top) and by a commercial antibody that recognizes the C-terminus of Int6 (middle). Actin was the loading control (bottom).

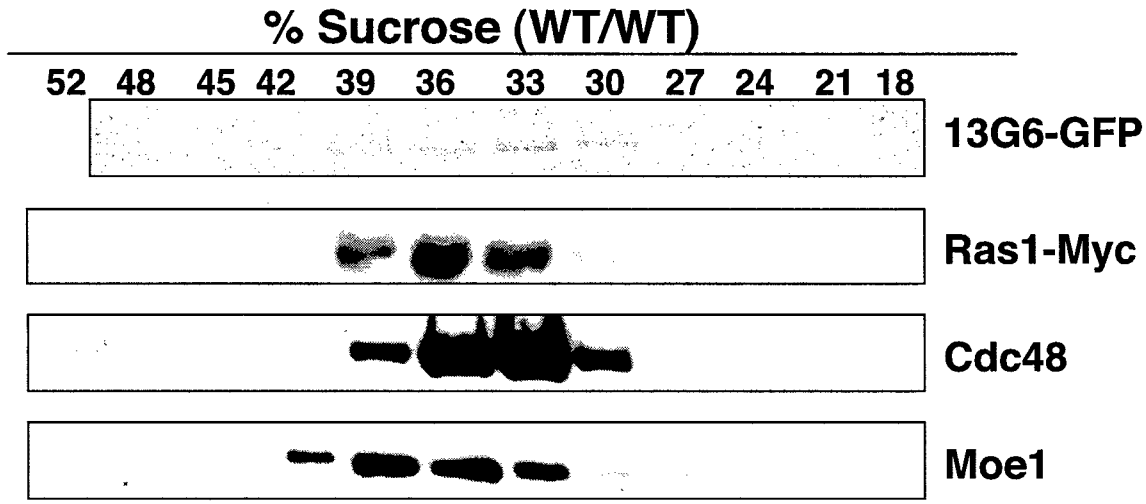


Figure 5. Moe1 and Ras1 co-fractionated with ER marker proteins. *S. pombe ras1* null cells were transformed with a vector expressing a mutagenized MYC-tagged Ras1 that localizes specifically to the ER, and this mutant Ras selectively signals down the Cdc42 pathway (submitted). This cell also contains an ER resident protein, 13G6, that is GFP-tagged. Cell lysates were fractionated by the sucrose gradient centrifugation and the resulting samples were analyzed by Western blots. Cdc48 binds polyubiquitinated proteins and Moe1 (data not shown) and it may present polyubiquitinated substrates via the Int6-Moe1 complex to the proteasome for degradation.